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# Multicellular-Vesicle-Promoting Polypeptide From *Trichoplusia ni:* Tissue Distribution and N-Terminal Sequence

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An N-terminal amino acid sequence of a 16.9 kDa hemolymph polypeptide, "Vesicle Promoting Factor" (VPF) from *Trichoplusia ni,* revealed a high sequence homology (70%) with *Manduca sexta* apolipophorin-III. A polyclonal antibody developed against VPF, however, was not immunoreactive with either purified *M. sexta* or *T. ni* apolipophorin-III. Immunoblots of tissue homogenates of *T. ni* indicated that VPF was present in imaginal wing discs, central nervous system (CNS), silk glands, midgut and hemocytes from fifth instar larvae, and also in the IAL-TND1 cell line which can grow as either fluid-filled multicellular vesicles or multicellular aggregates. VPF was also detected immunologically in the hemolymph of adults of *T. ni,* and in hemolymph of adults and larvae of *Galleria mellonella* and *Heliothis virescens.* Testes, midgut, hemocytes, and wing discs, but not Malpighian tubules, of *T. ni* released VPF into tissue culture medium during a 3 h incubation period. 

**Key words: cell line, development, *Manduca sexta,* apolipoprotein III, polypeptide, vesicles, *Trichoplusia ni,* wing discs**

## INTRODUCTION

Hormonally responsive cell lines from lepidopteran wing imaginal discs provide defined systems for fundamental studies on insect development (Oberlander and Miller, 1987). One such cell line, IAL-TND1, was developed from imaginal wing discs of the cabbage looper moth, *Trichoplusia ni* (Lynn et al., 1982). As has been observed for a number of insect cell lines of epithelial origin, the IAL-TND1 cells grew as fluid filled multicellular vesicles (Oberlander and Lynn, 1982). In addition it has been observed

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that lepidopteran pupal wings form such vesicles (Willis and Hollowell, 1976; Oberlander et al., 1987). Surprisingly, the IAL-TND1 vesicle morphology was stable only during the first year of culture, and the cells underwent a spontaneous change to an aggregate form. However, the process could be reversed, and the aggregates recovered the vesicular growth morphology by adding larval hemolymph to the aggregate cultures (Lynn et al., 1985). The vesicle promoting activity was in turn inhibited by the simultaneous presence of 20-hydroxyecdysone in the culture medium (Oberlander et al., 1987).

Thus, the dramatic effect of "Vesicle Promoting Factor" (VPF) on morphogenesis of a wing imaginal disc derived cell line in vitro, as well as its inhibition by 20-hydroxyecdysone, suggests that a role in influencing wing development in vivo must be considered. As a first step in understanding the action of VPF we utilized gel permeation and polyacrylamide gel electrophoresis and chromatofocusing techniques to identify a 16.9 kDa polypeptide from larval hemolymph which possessed VPF activity (Ferkovich et al., 1987). In the present work we have analyzed VPF with respect to its N-terminal amino acid sequence and distribution among larval tissues in vivo.

## MATERIALS AND METHODS

### Insects and Tissue Preparation

Insects were reared and staged as described by Leppla et al. (1984). Tissues were dissected from staged fifth instar larvae in Grace's tissue culture medium (GIBCO, Grand Island, NY).

### Purification of VPF and Sequence Analysis

VPF was isolated from larval hemolymph using a published procedure (Ferkovich et al., 1987) which included gel permeation chromatography and isoelectric focusing (IEF). VPF was eluted from the IEF gel bed (isoelectric point = pH 6.21) with 2 × Ringer's solution, and was dialyzed and concentrated against 1 × Ringer's to 1 mg/ml using a Pro DiMem® unit with 10 k membrane (Bio-Molecular Dynamics, Beaverton, OR). This fraction tested positively for VPF activity in a bioassay described earlier (Ferkovich et al., 1987). Samples were prepared for gel electrophoresis in Laemmli sample buffer (LSB, 1:1 v/v) and run on 15% SDS gels (Laemmli, 1970). Molecular weight standards were obtained from Bio-Rad (Hercules, CA): phosphorylase B, serum albumin, bovine muscle actin, carbonic anhydrase, trypsin inhibitor, and lysozyme. The SDS gel was blotted using a Trans-Blot Cell unit (Bio-Rad) onto a glass fiber filter. The VPF band was partially sequenced and amino acid analysis was performed by the Protein Core Facility, University of Florida, Gainesville, FL. The amino acid analysis (with loss of tryptophan) was performed on a Beckman 6300 Amino Acid Analyzer (Beckman Instruments, Fullerton, CA) according to Deutscher (1990). The N-terminal amino acid sequence was determined by automated Edman degradation using an online sequencing analyzer (Model 470A, Applied Biosystems, Foster City, CA) as described by Walker (1984).

### Carbohydrate Analyses

A sample that contained IEF-purified VPF was separated on a 17.5% SDS minigel (Bio-Rad), blotted onto nitrocellulose. The transfer membrane was washed in "A" buffer (10 mM Tris/HCl, pH 7.4, 500 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% $NaN_3$) containing 0.05% Tween-20® for 1 h. The blot was then washed 2 × 30 min in buffer A, incubated for 45 min in buffer A with 1 mg/ml Con A-FITC (Sigma Chemical Co., St. Louis, MO), washed 3 × 10 min in buffer A, and examined under UV (254 nm) for evidence of fluorescing glycosylated proteins.

### Biotinylated Lectin

Lectin detection of glycoproteins was carried out according to Dunbar (1987). The transfer membrane was washed in TBS buffer (10 mM Tris/HCl, pH 7.4, 155 mM NaCl) for 10 min, then incubated in deglycosylated bovine serum albumin (Sigma) for 2 h at 25°C with gentle agitation. The membrane was rinsed 2 × 20 min in TBS, and incubated for 3 h with gentle agitation in a 1:100 dilution of biotinylated lectin: biotin labeled succinyl-concanavalin A (Sigma). The lyophilized succinyl-con A powder was previously hydrated in 50 mM Tris/HCl, pH 7.5, 0.02% $NaN_3$ at 1 mg/ml (final dilution 1:10,000 w/v). The transfer membrane was then washed 2 × 20 min in TBS, incubated for 3 h in 1:200 avidin-conjugated horseradish peroxidase, rinsed again 2 × 20 min, and placed in substrate solution for development.

### Purification of Apolipophorin III

Apolipophorin III was purified from hemolymph obtained from *Heliothis virescens* and *T. ni* according to Wells et al. (1985). Briefly, hemolymph was centrifuged at 13,000*g* for 5 min to remove the hemocytes. The hemocyte-free hemolymph was then subjected to gel permeation on Sephadex G-75, followed by affinity chromatography using concanavalin A-Sepharose.

### Cell Culture and Assay

The cell line, IAL-TND1, derived from imaginal wing discs of *T. ni*, was used to assay vesicle-promoting activity of hemolymph and other tissues as described earlier (Ferkovich et al., 1987). Tissue culture multiwell plates were maintained at 26°C, and multicellular vesicles were counted after 6 days of exposure to test media. Specific activity is expressed as the number of vesicles/μg protein in a test well minus the number of vesicles/μg protein in the control well.

### Immunochemistry and VPF Presence in Larval Tissues

VPF samples purified by IEF were applied to a 3 mm preparative polyacrylamide gel, 17.5% SDS using a Protean® unit (Bio-Rad). The gel was run at 10–13 mA constant current and 480 V maximum, then at 40 mA to the end of the run. A 7 mm reference strip was cut from the gel and stained for 15 min in 0.1% R-250 Coomassie Blue stain in methanol:acetic acid (40:7%). The section of the preparative gel that corresponded to VPF was excised and macerated 4 × using 10 cc syringes and then used to raise antiserum in rabbits (Dunbar, 1987).

The antiserum was purified using a HIPac protein A column (Chromatochem, Missoula, MT). Three ml of serum was applied to the column in adsorption buffer: 10 mM sodium phosphate, pH 7.4. Bound IgGs were eluted using 100 mM Tris/HCl, pH 8, and were monitored at 280 nm. The VPF reactive fraction was then concentrated using a 10 K ProDiMem dialysis membrane (Bio-Molecular Dynamics) against 50 mM Tris/HCl pH 7.5, 20 mM NaCl, 0.075% $NaN_3$ at 4°C. At a 1:670 dilution, the purified antiserum gave a strong monospecific reaction with the VPF band in immunoblots of hemolymph separated on 17.5% SDS gels using blotting grade Protein A horseradish peroxidase (HRP) conjugate (Bio-Rad) (Towbin et al., 1979).

Imaginal discs and central nervous system (CNS) from staged *T. ni* were dissected directly into 1 × LSB; whereas fat body, silk glands, midgut, and hemocytes were collected in homogenization buffer (HB): 50 mM Tris/HCL, pH 8.0, 1 mM phenylmethylsulphonyl fluoride, 1 mM ethylenediaminetetraacetic acid, and 0.01% 1-phenyl-2-thiourea. Vesicles and aggregates from 1 culture flask were allowed to settle out (250 µl) and were rinsed 3 × with 125 µl HB. The tissues were then sonicated 3 × for 10 sec on ice, all samples were microfuged 5 min at 13,000*g*, and the supernatants were collected. Hemolymph was diluted to 16% (v/v) and conditioned media to 10% (v/v) HB. Aliquots of 30 µl of each preparation were added to 10 µl of 4× LSB and then heated at 95°C for 5 min. The remaining portion of the sample was used to determine the total protein present according to Bradford (1976). The proteins were resolved by electrophoresis on 17.5% SDS gels and immunoblots of the gels were made.

## RESULTS

### VPF Structural Analyses

Edman degradation of purified *T. ni* VPF yielded an unambiguous sequence of 46 N-terminal amino acids (Fig. 1). Surprisingly, the deduced sequence was found to be identical in 33 of these positions to the mature N-terminus of the *M. sexta* apolipophorin-III polypeptide determined by sequence analysis of a cloned cDNA (Cole et al., 1987). As was found to be the case with apolipophorin-III comparisons between *M. sexta* and *Locusta migratoria* (Kanost et al., 1988), VPF showed little homology with the latter molecule.

The remainder of the 16.9 kDa VPF is also likely to be similar to *M. sexta* apolipophorin-III since the two amino acid compositions are substantially congruent (Table 1). With the exception of cysteine, valine, phenylalanine, and lysine, the mole percent values for the two proteins was within approxi-

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M. sexta   DAPAGGNAFEEMEKHAKEFQKTFSEQFNSLVNSKNTQDFNKALKDGSD
           ...       .  .... .. ..... ..........   ....... .
           ...       .  .... .. ..... ..........   ....... .
T. ni      DAP--PSPLEDIEKHAAEFEKTFSEEFNSLVNSKNTEELNKALKDGED
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Fig. 1. Comparison of the amino-termini of apolipophorin-III from *M. sexta* hemolymph with VPF from *T. ni* hemolymph. Amino acids are designated with one-letter codes.

**TABLE 1. Comparison of Amino Acid Composition of VPF From *T. ni* Hemolymph With Apolipophorin-III From *Manduca sexta* Hemolymph**

| | % of Total | |
|---|---|---|
| Amino acid composition | VPF[a] | ApolipoLp-III[b] |
| Cysteine | 3.2 | 0.0 |
| Aspartic acid and aspargine | 8.9 | 11.9 |
| Threonine | 3.8 | 4.8 |
| Serine | 4.5 | 7.8 |
| Glutamic acid and glutamine | 18.0 | 18.6 |
| Proline | 1.1 | 1.8 |
| Glycine | 2.5 | 2.9 |
| Alanine | 11.0 | 13.8 |
| Valine | 0.7 | 5.9 |
| Isoleucine | 0.9 | 1.2 |
| Methionine | 0.3 | 1.2 |
| Leucine | 7.8 | 7.2 |
| Tyrosine | 0.7 | 0.6 |
| Phenylalanine | 0.7 | 4.8 |
| Histidine | 1.4 | 2.3 |
| Lysine | 8.6 | 13.8 |
| Arginine | 0.7 | 1.2 |

[a]Based on an MW of 16.9 kDa with approximately 154 amino acids.
[b]Based on an MW of 17.0 kDa with approximately 167 amino acids from Kawooya et al. (1984).

mately 20% of each other. Like apolipophorin-III, VPF was found not to contain high mannose carbohydrate moieties in lectin-blotting assays (data not shown).

### Immunoreactivity of VPF Antiserum With *T. ni* Tissues

The polyclonal antiserum raised to VPF was used to determine the tissue distribution of VPF by Western blotting. An immunoreactive response to a band in the VPF region was detected in *T. ni* hemolymph throughout the entire 4th and 5th larval instars (Fig. 2). The VPF polypeptide was also detected in homogenates of wing imaginal discs and fat body (Fig. 3). Both larval fat body and CNS tissue produced a weak response, but also exhibited cross-reactivity with unidentified high molecular proteins.

### Metabolic Radiolabelling of VPF

Although VPF could not be discerned in the autoradiograph of labelled proteins in tissues homogenized after labelling with [$^{35}$S]-methionine (Fig. 4A), secreted proteins from seven out of the eight tissues tested were immunoreactive for VPF (Fig. 4B). The Malpighian tubules were the only nonreactive tissue. Surprisingly, secreted proteins from the aggregate and vesicle form of the cell line also immunoprecipitated with the VPF antiserum corresponding to VPF.

### Immunoreactivity of VPF Antiserum With Hemolymph of Other Species and Apolipophorin-III

Hemolymph from larvae and adults of *T. ni*, but not *G. mellonella* or *H. virescens*, contained VPF in a Western blot assay (Fig. 5). In larval and

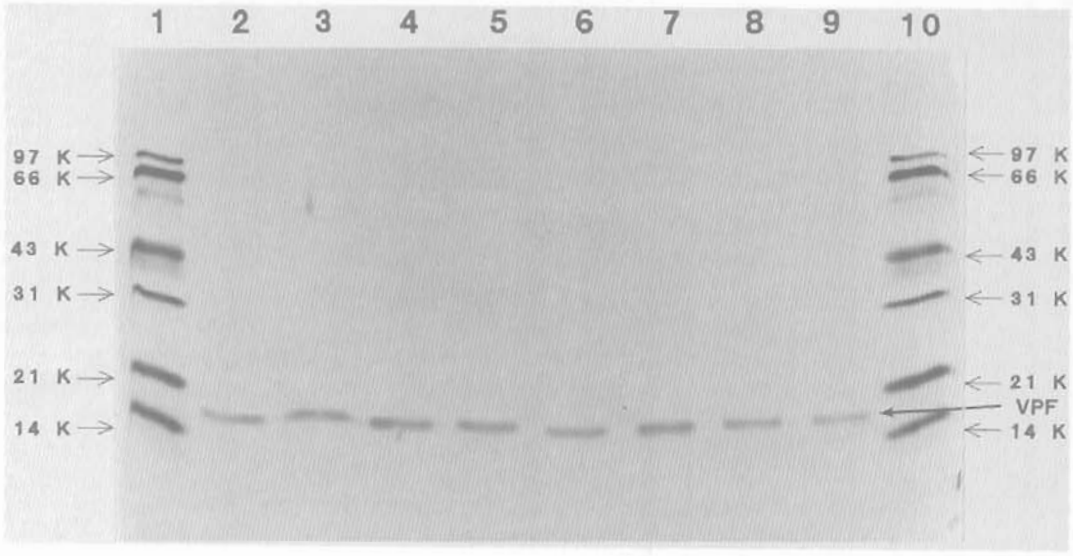


Fig. 2. An immunoblot of hemolymph from late 4th and early 5th instar larvae with VPF antiserum. Lanes: **(1,10)** protein standards; **(2)** late 4th instar, day 8; **(3)** late 4th instar, day 9; **(4)** late 4th instar (wandering and molt); **(5)** 5th instar, day 10; **(6)** mid 5th instar, day 11; **(7–9)** late 5th instar, day 12 (prewandering). Development was staged from day of oviposition ± 12 h, day 0.

adult hemolymph of *Galleria mellonella* and *Heliothis virescens*, unidentified high molecular weight polypeptides were again detected using the VPF antiserum.

Purified apolipophorin-III from *M. sexta* hemolymph did not cross-react with the VPF antiserum even though the two proteins had considerable sequence similarity in their N-termini. Moreover, apolipophorin-III from *M. sexta* as well as apolipophorin-III from *T. ni* and *H. virescens* failed

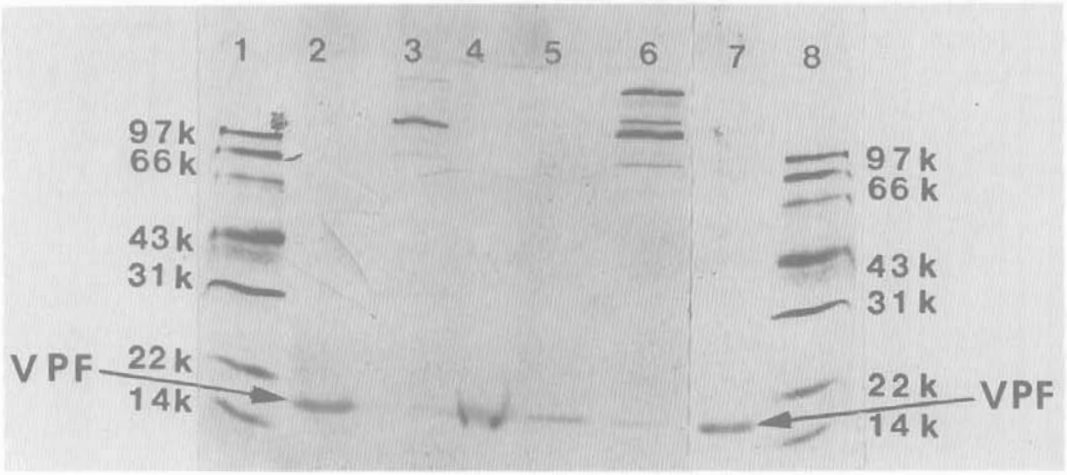


Fig. 3. Immunoblot of homogenized tissues from fifth instar larvae with VPF antiserum assayed with goat anti-rabbit HRP and protein A conjugates. Lanes: **(1,8)** protein standards; **(2,7)** hemolymph; **(3)** central nervous system; **(4)** wing discs; **(5)** hemocytes; **(6)** fat body.

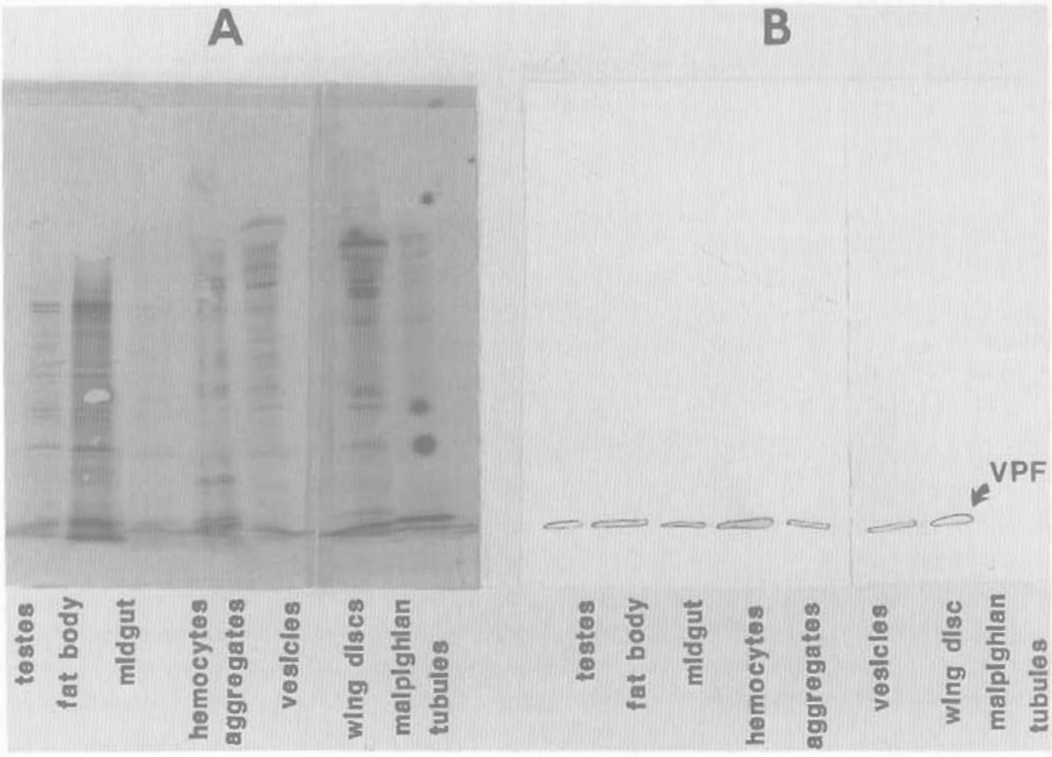


Fig. 4. Protein profile **(A)** and **(B)** using VPF antiserum of tissue proteins released into the Graces' tissue culture medium. Dissected tissues were incubated in medium for 3 h, then removed before applying samples of the medium on the SDS gels.

to induce vesicle formation when assayed at 5, 20, and 50 μg of protein per well. In contrast, 0.8 and 8 μg of VPF isolated from *T. ni* hemolymph induced 14 ± 4 and 263 ± 10 vesicles per well, respectively (data not shown).

## DISCUSSION

While our previous research with the IAL-TND1 cell line focused on its developmental properties in vitro, the present work dealt with the occurrence and tissue distribution of VPF in vivo. VPF was present in hemolymph throughout the 4th and 5th larval instars, as well as in male and female adults of *T. ni*. Glycoprotein analysis indicated that VPF did not bind concanavalin A and is, therefore, not likely to be glycosylated. Although we could not determine if VPF specifically was synthesized by various tissues incubated with [$^{35}$S]-methionine, VPF was detected in immunoblots of proteins secreted by seven of the eight tissues of *T. ni* tested. These findings agree with the results of a previous study that monitored VPF activity by co-culturing certain tissues from *T. ni* and other species with IAL-TND1 cells in the aggregate form and counting the number of multicellular vesicles produced (Lynn et al., 1985).

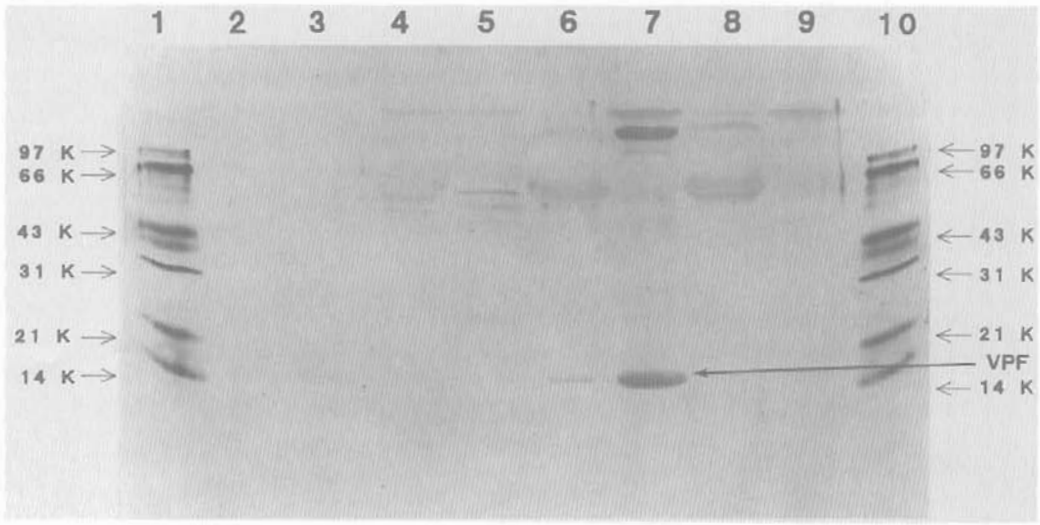


Fig. 5. Immunoblot of purified apolipophorin-III from *Manduca sexta* and hemolymph from larvae and adults of three lepidopteran species, assayed with VPF antiserum. Lanes: **(1,10)** protein standards; **(2,3)** apoLp-III; **(4)** *Galleria mellonella* larva; **(5)** *G. mellonella* adult; **(6)** *T. ni* larva; **(7)** *T. ni* adult; **(8)** *Heliothis virescens* larva; **(9)** *H. virescens* adult.

Our findings suggest that not only does VPF regulate morphogenesis of an imaginal wing disc-derived cell line, but also that its presence during postembryonic development in both the hemolymph and a variety of tissues presents the possibility of a role in vivo. Nevertheless, the widespread distribution by both stage and tissue does not preclude a significant role for VPF in wing development. Thus, it may be that VPF has a fundamental biochemical activity that becomes critical during wing imaginal disc morphogenesis.

In this connection we studied VPF's similarity with apolipoprotein-III, one of the three insect apolipoproteins which functions in the lipid binding activity of lipophorin in the hemolymph of *M. sexta* (Cole et al., 1987). The N-terminal amino acid sequence of VPF had a significant homology with that of apolipophorin-III, and VPF similarly exists free in the hemolymph and appears to be non-glycosylated. However, VPF is dissimilar to apolipophorin-III in that VPF contains cysteine and apolipophorin-III does not (Kawooya et al., 1984). In addition VPF was found not only in the hemolymph, but was also immunoreactive with a variety of tissues. Whether or not VPF in the hemolymph of *T. ni* has a similar function to the apolipoproteins in *M. sexta* is not known.

Although our results demonstrate VPF in vivo, its activity throughout the 4th and 5th instars and in adults and its presence in a number of tissues provide an obstacle to demonstrating any specificity with regard to wing disc development. Still, the actions in vitro on morphogenesis of the wing disc derived cell line, and the inhibition of VPF activity by ecdysteroids in vitro suggest that further efforts are warranted in determining the action of this molecule in vivo.

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